A p38^{MAPK}/HIF-1 Pathway Initiated by UVB Irradiation Is Required to Induce Noxa and Apoptosis of Human Keratinocytes

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The signal transduction pathways leading to apoptosis of human keratinocytes responding to UVB irradiation are complex and not completely understood. Previously, we reported that in UVB-irradiated keratinocytes, p38^{MAPK} instigates Bcl-2-associated X protein (Bax) activation and mitochondrial apoptosis. However, the molecular mechanism underlying the pro-apoptotic function of p38^{MAPK} remained unclear. Here, we show that in UVB-treated human primary keratinocytes the activation of p38^{MAPK} is necessary to upregulate Noxa, a BH3-only pro-apoptotic dominantly induced by UVB and required for apoptosis. Whereas p53-silencing was marginally cytoprotective and poorly affected Noxa expression, p38^{MAPK} inhibition in p53-silenced keratinocytes or in p53$^{-/-}$ cells could still efficiently prevent Noxa induction and intrinsic apoptosis after UVB, indicating that p38^{MAPK} signals mainly through p53-independent mechanisms. Furthermore, p38^{MAPK} was required for the induction and activation of hypoxia-inducible factor 1 (HIF-1) in response to UVB, and HIF-1 knockdown reduced Noxa expression and apoptosis. In UVB-irradiated keratinocytes, Noxa targeted the anti-apoptotic myeloid cell leukemia sequence 1 (Mcl-1) for degradation, and small-interfering RNA (siRNA)-mediated knockdown of Noxa or p38^{MAPK} inhibition restored levels of Mcl-1 and abolished apoptosis. Thus, the pro-apoptotic mechanisms orchestrated by p38^{MAPK} in human keratinocytes in response to UVB involve an HIF-1/Noxa axis, which prompts the downregulation of anti-apoptotic Mcl-1, thereby favoring Bax-mediated mitochondrial apoptosis of UVB-damaged keratinocytes.

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INTRODUCTION

The UV portion of sunlight is a ubiquitous stress to human skin and the steep rising incidence of nonmelanoma skin cancer during the last decade is a direct consequence of the increased exposure to this genotoxic and mutagenic agent. Although there is no doubt that long wave UVA (320–400 nm) can also contribute to skin cancer, most of the mutagenic and carcinogenic properties of sunlight have been attributed to UVB (290–320 nm) (Afaq *et al.*, 2005). Depending on the level of UVB-induced damage, keratinocytes can become growth arrested and repair the UVB-induced DNA lesions or, when irreparable damage is inflicted, apoptosis (''sunburn cell'' or SBC formation) is initiated to eradicate potentially mutagenic cells (de Gruijl *et al.*, 2001). Although UVB-induced apoptosis involves cascades triggered by direct DNA damage, reactive oxygen species production and ligand-independent receptor activation (Kulms and Schwarz, 2002; Assefa *et al.*, 2005; Herrlich *et al.*, 2008), several evidences have indicated that the intrinsic or mitochondrial pathway of apoptosis is the predominant mechanism in SBC formation (Grossman *et al.*, 2001; Takahashi *et al.*, 2001; Sitailo *et al.*, 2002).

Intrinsic apoptosis is engaged by a variety of stress signals disrupting the mitochondrial outer membrane, which results in the release of cytochrome *c* in the cytosol and activation of the apoptosome, the platform for caspase 9 activation (Green, 2005). This pathway is tightly regulated by B-cell lymphoma (Bcl-2) family members consisting of anti-apoptotic Bcl-2 proteins (e.g., Bcl-2, Bcl-x_L, Mcl-1), pro-apoptotic ''BH123 multidomain'' (e.g., Bax, Bak) and ''BH3-only'' proteins (e.g., Bid, Bad, Noxa, PUMA) (Youle and Strasser, 2008). Although there is no doubt that anti- and pro-apoptotic proteins thwart each others function, the molecular mechanisms underlying pro-apoptotic Bcl-2 proteins action appear to be different.

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Abbreviations: Ask-1, apoptosis signaling kinase 1; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma; Bcl-x_L, B-cell lymphoma-extra large; Bim, Bcl-2-interacting mediator of cell death; BNIP3, Bcl-2/adeNovirus E1B 19 kDa protein-interacting protein 3; HIF-1, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; Mcl-1, myeloid cell leukemia sequence 1; NHK, normal human keratinocyte; PARP, poly (ADP-ribose) polymerase; PUMA, p53 upregulated modulator of apoptosis; SBC, sunburn cell; SCC, squamous cell carcinoma

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Genetic and biochemical evidence indicate that when activated by different signaling pathways pro-apoptotic ''BH3-only'' proteins act predominantly by binding to and neutralizing the activity of the anti-apoptotic Bcl-2 proteins, thereby unleashing Bcl-2-associated X protein (Bax) and Bak from their negative control and allowing their oligomerization/activation, which results in mitochondrial membrane permeabilization (Lomonosova and Chinnadurai, 2008; Youle and Strasser, 2008).

Recently, the BH3-only family member Noxa has been identified as the dominant regulator of UVB-induced apoptosis, because loss of Noxa severely suppressed SBC formation in UV-irradiated mice (Naik *et al.*, 2007). Several DNA damaging agents and apoptotic inducers have been shown to upregulate Noxa through p53-dependent mechanisms (Villunger *et al.*, 2003; Ploner *et al.*, 2008). Indeed, it is well established that p53 accumulates rapidly in keratinocytes responding to UVB-mediated genotoxic damage and influences apoptotic induction (Benjamin and Ananthaswamy, 2007). Furthermore, the role p53 has in skin cancer is shown by the observations that skin tumors frequently harbor UVB-induced p53 mutations and that p53 null mice display increased incidence of skin cancer (Ziegler *et al.*, 1994; Bruins *et al.*, 2004). However, UVB-induced apoptotic signaling also entails p53-independent pathways, as p53-negative cells or keratinocytes with mutated p53 still undergo UVB-induced apoptosis (Assefa *et al.*, 2005).

Recently, HIF-1 (hypoxia-inducible factor-1) has also been suggested to have a role in UVB-induced apoptosis in human keratinocytes (Rezvani *et al.*, 2007). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor consisting of an inducible α (HIF-1α) subunit and a β (HIF-1β or aryl hydrocarbon receptor nuclear translocator) subunit (Semenza, 2007), whose expression is rather stimulus independent. HIF-1α-inducing conditions, such as hypoxia, have been shown to increase its expression and prevent its ubiquitinylation and subsequent proteasomal degradation, thereby promoting HIF-1 stabilization and activation. HIF-1 regulates an extensive set of genes, involved mainly in cytoprotection and angiogenesis, but also apoptosis (Piret *et al.*, 2002). For instance, HIF-1 has been shown to mediate hypoxic cell death by induction of pro-apoptotic BH3-only proteins Bcl-2/adeNovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) (Kothari *et al.*, 2003) and Noxa (Kim *et al.*, 2004). The main targets of the stress-responsive HIF-1 in normoxic conditions are less characterized and, more precisely, for UVB have not been revealed yet.

Recently we showed that in human keratinocytes UVB-mediated Bax activation and mitochondrial apoptosis is governed by the Ask1-MKK3/6-p38^{MAPK} signaling cascade (Van Laethem *et al.*, 2004, 2006). However, the molecular mechanism by which activation of p38^{MAPK} in UVB-treated cells instigates Bax-mediated intrinsic apoptosis has not been clarified yet.

In this study, we hypothesized that p38^{MAPK} could be a major upstream regulator of Noxa in UVB-treated human keratinocytes and addressed the potential involvement of p53- and/or HIF-1-dependent mechanisms in this response. We show that p38^{MAPK} is required for Noxa induction, myeloid cell leukemia sequence 1 (Mcl-1) degradation, and intrinsic apoptosis after UVB, through the activation of HIF-1, whereas p53 seems to be largely dispensable.

RESULTS

Noxa upregulation after UVB is p38^{MAPK} dependent and required for apoptosis

We set out to investigate whether p38^{MAPK} exerts its pro-apoptotic role through the modulation of Noxa expression in UVB-irradiated normal human keratinocytes (NHKs).

To this end, we first monitored the kinetics of p38^{MAPK} activation, p53 and Noxa induction after UVB irradiation of NHKs (Figure 1a). Exposure of NHKs to a UVB dose of 120 mJ cm^{-2} prompted the apoptotic pathway as evidenced by caspase 3, poly (ADP-ribose) polymerase (PARP) cleavage (Figure 1A) and the accumulation of fragmented DNA (Figure 1b), ultimately resulting in 41 ± 2% of apoptotic cells after 24 h (Figure 1c).

Within the evaluated time frame, Noxa was nearly undetectable and p53 level was low in untreated NHKs, whereas in UVB-treated NHKs p53 and Noxa levels steadily increased to reach a maximal level 16–24 h after treatment

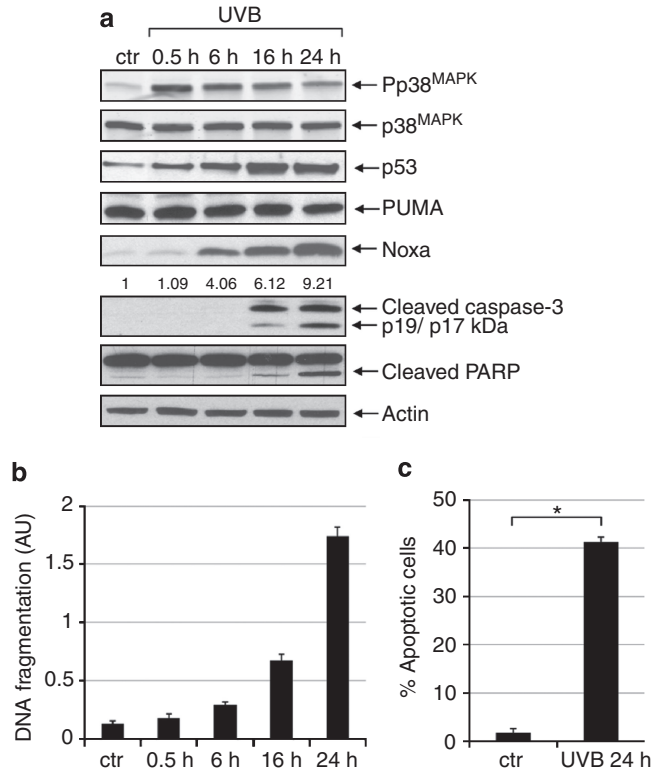

Figure 1. General UVB-induced response in normal human keratinocyte (NHK). NHKs were irradiated with a UVB dose of 120 mJ cm^{-2}. (**a**) Time-dependent western blot (WB) detection of p38^{MAPK} phosphorylation and protein level of p53, PUMA, Noxa (densitometric quantification of Noxa vs loading control of the corresponding WB analysis is also shown), cleavage of caspase 3, and poly (ADP-ribose) polymerase (PARP). Cell lysates were made and assays were performed at the indicated time points as detailed in Materials and Methods section. (**b**) DNA fragmentation (Cell Death ELISA) and (**c**) FACS analysis for sub-G1-positive cells (propidium iodide stained) in NHKs after UVB irradiation. The graph represents the mean ± standard deviation of three independent experiments.

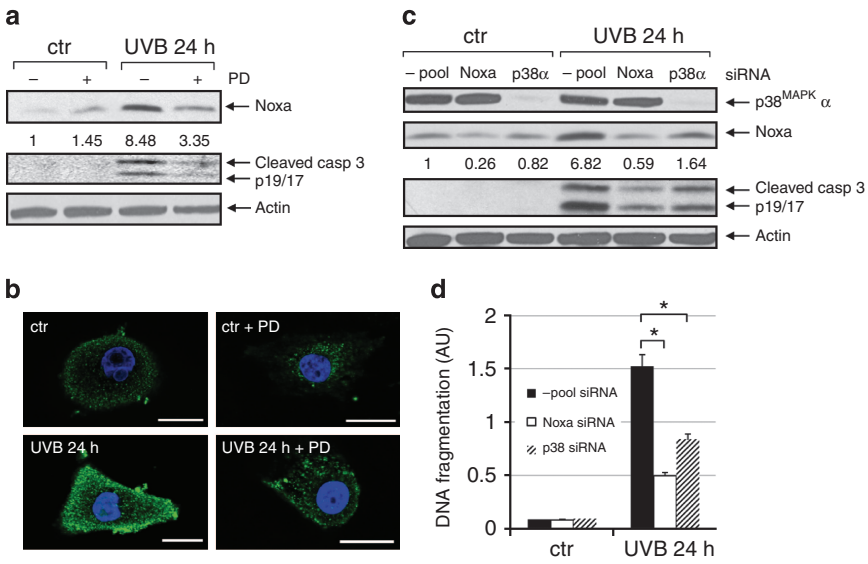

Figure 2. Noxa induction after UVB is p38^{MAPK}-dependent. (**a**) Western blot (WB) analysis for Noxa and cleaved caspase 3 and (**b**) confocal microscopy analysis of untreated (ctr) or UVB (120 mJ cm^{-2})-treated normal human keratinocytes (NHKs) immunostained for Noxa with or without 5 μM PD169316 (PD). Nuclei are DAPI-counterstained and the white scale bar in the lower right corner represents 20 μm; (**c**) WB analysis for p38^{MAPK}, Noxa, and cleavage of caspase 3. Quantification of Noxa versus loading control of the corresponding WB analysis is shown. (**d**) DNA fragmentation of NHKs transfected with scrambled-siRNA (-pool), Noxa-siRNA, or p38^{MAPK}α-siRNA. Lysates, pictures, and assays were made/performed at the indicated time points. Inhibitor was added 1.5 h and cells were siRNA-transfected 72 h before irradiation. The graph represents the mean ± SD of three independent experiments. Asterisk (*) indicates $P<0.05$ using the Student's *t*-test.

(Figure 1a). UVB did not seem to affect the protein levels of p53 upregulated modulator of apoptosis (PUMA), another BH3-only protein known to have a major role in apoptotic induction after various forms of genotoxic stress (Michalak *et al.*, 2008). As already shown in our previous work (Van Laethem *et al.*, 2004), p38^{MAPK} was rapidly activated after UVB irradiation, clearly preceding the induction of Noxa and the onset of apoptotic cell death (Figure 1a).

Because Noxa protein expression was clearly elevated at 24 h after UVB exposure in parallel to caspase 3 cleavage and DNA fragmentation (Figure 1a, b), we focused mainly on this time point and further investigated whether Noxa induction was p38^{MAPK} dependent. To this end, we evaluated the impact of p38^{MAPK} inhibition on Noxa protein expression and UVB-induced apoptosis either by using the p38^{MAPK}α/β small molecule inhibitor PD169316 or by small-interfering RNA (siRNA)-mediated knockdown of p38^{MAPK}α. Both inhibitory strategies resulted in a clear prevention of Noxa induction by UVB as judged by western blot analysis (Figure 2a, c) and immunocytochemistry (Figure 2b). Moreover, in conformity with previous studies (Shimizu *et al.*, 1999; Van Laethem *et al.*, 2006), p38^{MAPK} inhibition resulted in a significant reduction of DNA fragmentation and caspase 3 cleavage after UVB. Importantly, knockdown of Noxa also resulted in a severe reduction of apoptosis after UVB (Figure 2c and d), thus establishing a functional link between p38^{MAPK} and Noxa in SBC formation.

p38^{MAPK}-mediated induction of Noxa after UVB is largely p53 independent

Pretreatment of NHKs with the inhibitors of transcription and translation, i.e., actinomycin D and cycloheximide, completely blocked Noxa induction by UVB (data not shown), thus indicating that a transcriptional mechanism was involved.

Therefore, we next wondered which p38^{MAPK}-regulated transcription factor was involved. A primary candidate was p53, because Noxa is a known p53 target (Ploner *et al.*, 2008) and p38^{MAPK} regulates p53 stability and activity after UVB irradiation (Assefa *et al.*, 2005).

To investigate this possibility, we first silenced p53 expression in NHKs and evaluated the level of Noxa and UVB-induced apoptosis. Whereas siRNA-mediated knockdown of p53 completely blocked p53 accumulation by UVB (Figure 3a), it had only a modest influence on UVB-induced apoptosis (Figure 3b). Likewise, p53 knockdown poorly affected Noxa induction and caspase 3 cleavage after UVB irradiation (Figure 3a), thus suggesting that in our experimental setup, p53 contributes only to a minor extent to UVB-induced apoptosis in NHKs.

To further clarify the mechanisms of Noxa induction by UVB, we blocked p38^{MAPK} in p53-silenced NHKs. Figure 3a shows that even in p53-silenced NHKs, p38^{MAPK} inhibition decreased Noxa induction and prevented caspase 3 cleavage and apoptotic cell death (Figure 3b) in response to UVB. Likewise, as shown by immunofluorescence analysis (Figure 3c), blockage of p38^{MAPK} in p53-silenced UVB-treated NHKs decreased the number of cells displaying Bax activation (red-fluorescent staining indicating the conformational-active Bax), cytochrome *c* release (indicated by the green-fluorescent staining that becomes diffuse when cytochrome *c* is released from the mitochondria into the cytosol), and nuclear fragmentation (indicated by the blue nuclear staining), as compared to p53-silenced UVB-treated NHKs with intact p38^{MAPK} signaling.

Moreover, we used a p53-null squamous cell carcinoma (SCC) line (A253-p53$^{-/-}$) and reconstituted wt p53 by retroviral transduction (A253-p53$^{+/+}$), which resulted in

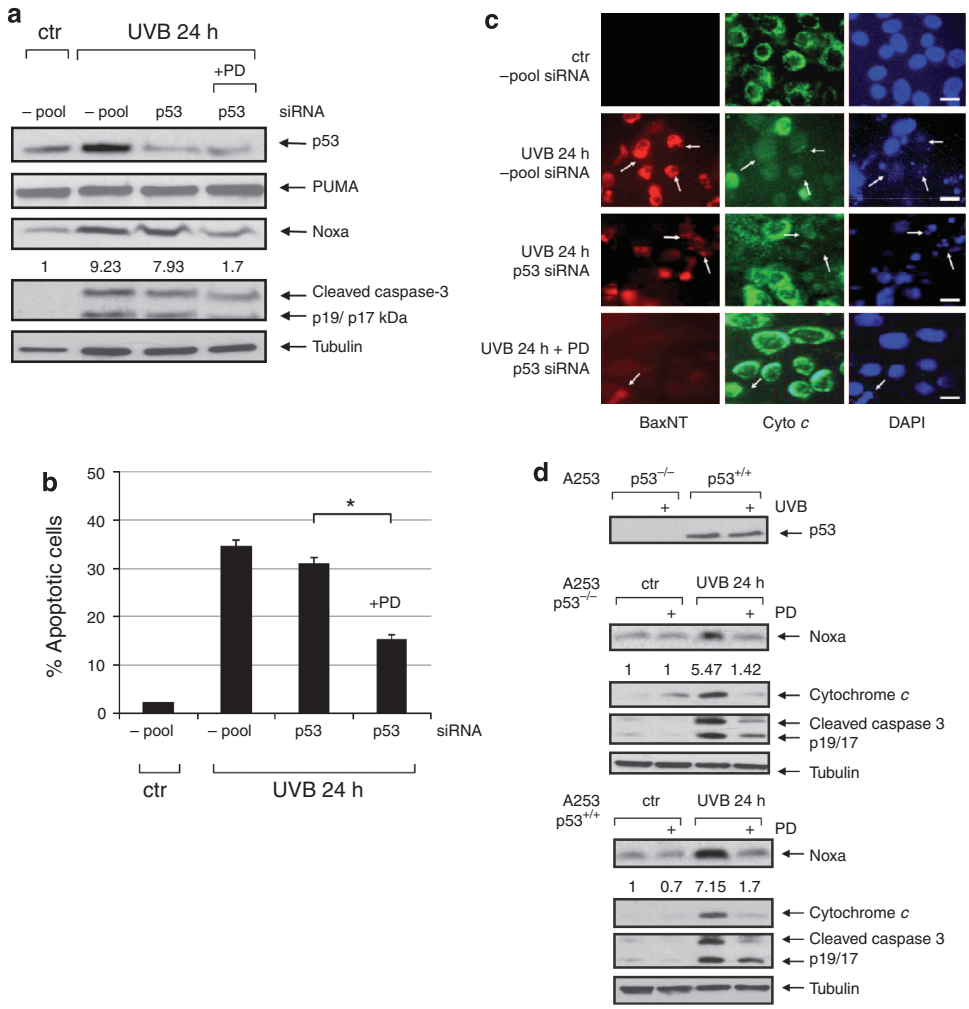

Figure 3. p38^{MAPK}-mediated Noxa induction is largely p53 independent. (**a**) Western blot (WB) analysis for p53, Noxa (+ quantification of Noxa/loading control), PUMA, and cleaved caspase 3; (**b**) FACS analysis for sub-G1-positive cells with/without PD169316 (5 μM; added 1.5 h before treatment); (**c**) immunofluorescence analysis of BaxNT (anti-active Bax antibody), cytochrome *c*, and nucleus (DAPI) in untreated (ctr) or UVB (120 mJ cm^{-2})-treated normal human keratinocytes (NHKs) transfected (72 h before treatment) with scrambled-siRNA (-pool) or p53-siRNA. Arrows indicate apoptotic cells; white scale bar in the right corner represents 20 μm; (**d**) WB for p53, Noxa (+ quantification of Noxa/loading control), cytosolic cytochrome *c*, and cleaved caspase 3 in p53$^{-/-}$ and p53$^{+/+}$ A253 cells. Lysates, pictures, and assays were made/performed at the indicated time points. Graphs represent mean ± SD of three independent experiments. Asterisk (*) indicates $P<0.05$ using the Student's *t*-test.

a stable expression of nuclear p53 (Figure 3d). In both cell lines, UVB irradiation led to comparable Noxa induction, cytochrome *c* release, and caspase 3 cleavage. Importantly, all these apoptotic parameters were equally well inhibited by PD169316 (Figure 3d), thus suggesting that p38^{MAPK} regulates Noxa and UVB-induced apoptosis largely through p53-independent mechanisms.

HIF-1 is essential for p38^{MAPK}-mediated Noxa induction and SBC formation

Because HIF-1 has been reported to regulate Noxa expression under hypoxic conditions (Kim *et al.*, 2004), we asked whether HIF-1 was required for Noxa induction in UVB-exposed keratinocytes and whether this process was dependent on p38^{MAPK}.

The induction of HIF-1α and subsequent increase in DNA-binding activity of HIF-1 after UVB in NHKs were observed peaking at 10–12 h after UVB (Figure 4a, b). To evaluate the role of HIF-1 in UVB-mediated Noxa induction and apoptosis, we silenced HIF-1 expression. Transfection of NHKs with scrambled (-pool) siRNA did not affect UVB-mediated HIF-1α accumulation, as expected, whereas NHKs transfected with siRNA targeting HIF-1α-specific sequences (Figure 4c) were impaired in their ability to mount HIF-1α protein level after UVB. Interestingly, HIF-1 knockdown inhibited Noxa induction by UVB, both at the mRNA (Figure 4d) but especially at the protein levels, whereas BNIP3, a known HIF-1 target, was not affected (Figure 4e). Importantly, inhibition of HIF-1 expression also blocked caspase 3 cleavage (Figure 4e) and DNA fragmentation (Figure 4f) after UVB, thus suggesting that Noxa is a pro-apoptotic target of HIF-1 in UVB-irradiated NHKs.

Because UVB-induced HIF-1α upregulation and HIF-1 DNA-binding activity followed a biphasic modulation (Rezvani *et al.*, 2007), we decided to evaluate the role of p38^{MAPK} in this process when HIF-1α induction after UVB

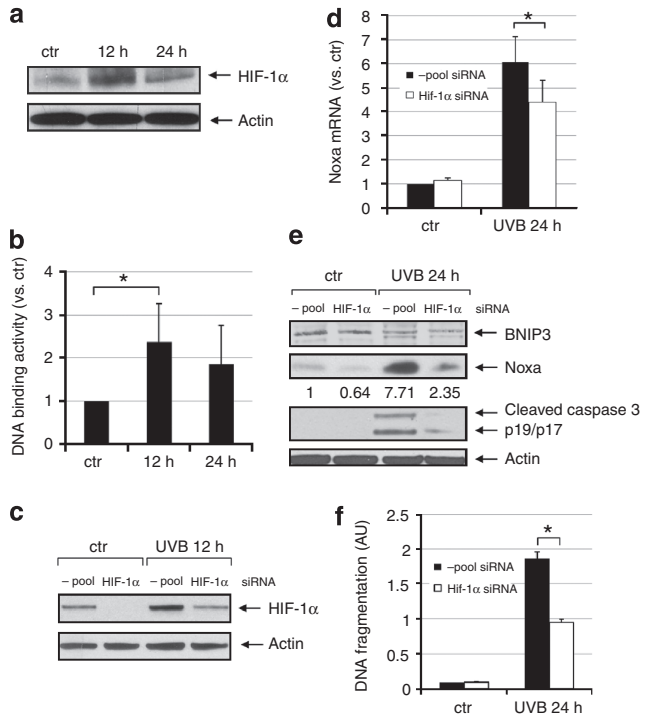

Figure 4. Noxa upregulation after UVB is hypoxia-inducible factor 1 (HIF-1) dependent. (**a**) Western blot (WB) detection of HIF-1α and (**b**) HIF-1 DNA-binding activity (ELISA) in untreated (ctr) or UVB-treated normal human keratinocytes (NHKs). (**c**) WB analysis for HIF-1α; (**d**) Q-PCR analysis of Noxa mRNA levels; (**e**) BNIP3, Noxa (densitometric quantification of Noxa vs loading control of the corresponding WB analysis is shown), and cleaved caspase 3; and (**f**) DNA fragmentation (Cell Death ELISA) in untreated (ctr) or UVB-treated NHKs transfected with scrambled-siRNA (-pool) or HIF-1-siRNA. Lysates were made and assays were performed at the indicated time points as detailed in Materials and Methods section. NHKs were irradiated with a UVB dose of 120 mJ cm^{-2} and were siRNA-transfected 72 h before irradiation. Graphs represent the mean ± standard deviation of three independent experiments. Asterisk (*) indicates $P<0.05$ using the Student's *t*-test.

reached a plateau, i.e., 10–12 h. Inhibition of $p38^{MAPK}$ drastically decreased UVB-induced HIF-1α upregulation and HIF-1 DNA-binding activity (Figure 5a and b). In addition, the knockdown of $p38^{MAPK}\alpha$ prevented the accumulation of HIF-1α protein (Figure 5c) in UVB-irradiated NHKs.

Thus, HIF-1 activity in UVB-exposed NHKs is substantially $p38^{MAPK}$ dependent and Noxa is a downstream element of this signaling cascade.

UVB-induced Noxa upregulation causes Mcl-1 degradation and Bax activation

We then investigated how Noxa induction promoted mitochondrial apoptosis in UVB-exposed NHKs. To this end, we first evaluated the protein levels of different anti-apoptotic family members at different time points after UVB irradiation. Whereas Bcl-2 and B-cell lymphoma-extra large (Bcl-x_L) expression was not altered, Mcl-1 level progressively decreased after UVB irradiation (Figure 6a). The kinetic of UVB-induced Mcl-1 degradation correlated well with the induction of Noxa expression (Figure 1a), whereas PUMA (Figures 1 and 3a) or BNIP3 (Figure 4e) level did not change.

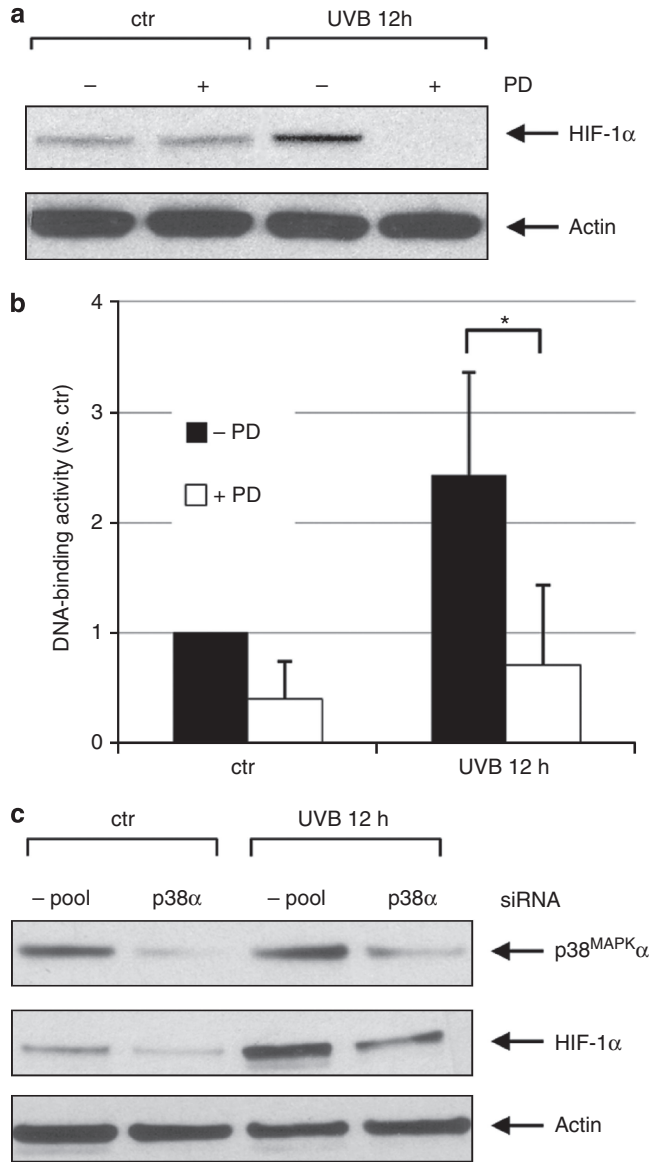

Figure 5. Hypoxia-inducible factor 1 (HIF-1) after UVB is $p38^{MAPK}$ dependent. (**a**) Western blot (WB) analysis of HIF-1α and (**b**) HIF-1 DNA-binding activity (TransAM HIF-1 activity ELISA) in untreated (ctr) or UVB (120 mJ cm^{-2})-treated normal human keratinocytes (NHKs) in the presence or absence of PD169316 (5 μM), (**c**) WB analysis of HIF-1α and $p38^{MAPK}\alpha$ of untreated (ctr) or UVB-treated NHKs transfected with scrambled siRNA (-pool) or $p38^{MAPK}\alpha$-siRNA. Lysates were made and assays were performed at the indicated time points as detailed in Materials and Methods section. Inhibitor was added 1.5 h and cells were siRNA-transfected 72 h before irradiation. The graph represents the mean ± standard deviation of three independent experiments. Asterisk (*) indicates $P<0.05$ using the Student's *t*-test.

To unravel a possible functional link between Mcl-1 and Noxa-mediated apoptosis, we blocked Noxa induction by UVB through siRNA-mediated knockdown. Inhibition of the basal Noxa expression level in untreated cells did not affect Mcl-1, whereas silencing Noxa induction by UVB restored Mcl-1 protein level (Figure 6b). Moreover, $p38^{MAPK}$ inhibition functionally mimicked the effect of Noxa knockdown and stabilized Mcl-1 after UVB irradiation (Figure 6c).

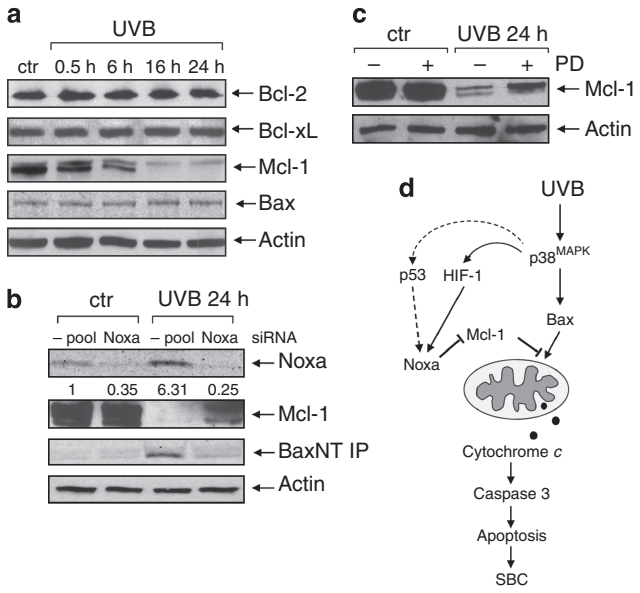

Figure 6. Myeloid cell leukemia sequence 1 (Mcl-1) degradation and Bcl-2-associated X protein (Bax) activation after UVB are Noxa dependent. (**a**) Time-dependent western blot (WB) analysis of B-cell lymphoma (Bcl-2), B-cell lymphoma-extra large (Bcl-x_L), Mcl-1, and Bax in NHKs after UVB (120 mJ cm^{-2}) irradiation. (**b**) WB analysis of Noxa (densitometric quantification of Noxa vs loading control of the corresponding western blot analysis is shown), Mcl-1, and immunoprecipitated BaxNT in untreated (ctr) or UVB-treated NHKs transfected with scrambled-siRNA (-pool) or Noxa-siRNA. (**c**) WB analysis of Mcl-1 in the presence or absence of PD169316 (5 μM) of untreated (ctr) or UVB-treated NHKs. (**d**) Schematic overview of the UVB-induced p38^{MAPK}-HIF-1-Noxa pathway. Lysates were made and assays were performed at the indicated time points as detailed in Materials and Methods section. Inhibitor was added 1.5 h and cells were siRNA-transfected 72 h before irradiation.

We immunoprecipitated Bax, with an antibody recognizing its activated conformation, from scrambled-siRNA (-pool) and Noxa-siRNA transfected NHKs before and after UVB irradiation. As shown in Figure 6c, although UVB exposure of NHKs (scrambled-siRNA or -pool) resulted in a clear increment of the fraction of active Bax as compared to untreated cells, in Noxa-silenced NHKs activated Bax levels after UVB were severely reduced. These differences were not due to Bax upregulation because Bax protein level remained unaltered (Figure 6a).

Thus, p38^{MAPK}-dependent Noxa induction after UVB is required to target Mcl-1 for degradation, leading to activation of Bax and the engagement of intrinsic apoptosis (Figure 6d).

DISCUSSION

This study shows that activation of p38^{MAPK} in UVB-treated NHKs is essential for the induction of the pro-apoptotic BH3-only Noxa, which launches intrinsic apoptosis through Mcl-1 downregulation. Furthermore, we provide evidence that p38^{MAPK} induces Noxa through an HIF-1-dependent mechanism, whereas p53 is largely dispensable.

It is well established that UVB-induced apoptosis proceeds through Bcl-2 inhibitable pathways (Assefa *et al.*, 2005), thus implicating mitochondria as central coordinators of cell death in keratinocytes. In this study, we found that among the BH3-only proteins known to be transcriptionally upregulated by p53, only the expression of Noxa is induced by UVB, whereas PUMA protein level remained unaffected. This is consistent with a recent study showing that after UVB irradiation, Noxa is the prominent BH3-only protein upregulated in cultured mouse embryonal fibroblasts and in keratinocytes of intact mouse skin undergoing apoptosis (Naik *et al.*, 2007).

Furthermore, we found that p53 knockdown in NHKs or p53 deficiency in A253 cells did not abrogate UVB-induced Noxa upregulation and poorly protected against UVB, thus suggesting p53-independent pathways of UVB-induced apoptosis. In line with this concept, although UVB-irradiated skin from p53$^{-/-}$ mice displays a significantly reduced number of apoptotic keratinocytes, SBC formation is not completely abated (Naik *et al.*, 2007). In addition, loss of Noxa has a more pronounced protective effect than the loss of p53 in UV-irradiated skin, thus indicating that p53-dependent pathways of apoptosis cannot solely explain SBC formation *in vivo*. Interestingly, these less-defined p53-independent pathways are overruled by Bcl-2 overexpression. Thus, it is tempting to assume that p38^{MAPK} is the key coordinator of those p53-independent signals, because the pro-apoptotic effects of the p38^{MAPK} cascade in UVB-exposed keratinocytes are completely inhibited by the overexpression of Bcl-2 (Van Laethem *et al.*, 2004). Moreover, p38^{MAPK} inhibition resulted in a marked protection against UVB-induced apoptosis independent of the cellular p53 status (this study and Van Laethem *et al.*, 2004).

The surprisingly limited contribution of p53 in UVB-induced apoptosis in our study might be explained by the fact that we have used cultured NHKs, which more closely resemble keratinocytes from the epidermal basal layer. These proliferating NHKs, in contrast to the mature differentiating keratinocytes of the suprabasal epidermis, express members of the p63 family (Reis-Filho *et al.*, 2002) thought to constitutively bind *in vivo* to p53-dependent promoters involved in apoptosis, thereby suppressing the access of p53 to its target genes (Ghioni *et al.*, 2002). This could reflect the finding that expression of pro-apoptotic p53-dependent factors such as Bax and PUMA remained unchanged, although p53 was clearly induced by UVB in NHKs. The observations that p53 knockdown in cultured NHKs can paradoxically increase UVB-induced apoptosis (Chaturvedi *et al.*, 2005), and Notch1 activity/expression in UVB-exposed skin is p53-dependent and exerts a pro-survival function by suppressing pro-apoptotic FoxO3a gene (Mandinova *et al.*, 2008), further unravels that p53 functions in the UVB response of human skin are not straightforward. This complexity is also emerging in other cellular paradigms where p53, besides apoptosis, has been shown to regulate survival, cellular redox state, and metabolism in a highly cell- and context-dependent way (Horn and Vousden, 2007).

This study further reveals a previously unreported transcriptional mechanism governed by p38^{MAPK} and required for the induction of Noxa. Consistent with a recently reported pro-apoptotic role of HIF-1 in UVB-irradiated NHKs

(Rezvani *et al.*, 2007), in this study we found that HIF-1 was activated by $p38^{MAPK}$ and was responsible for the induction of Noxa after UVB. In agreement with a pro-apoptotic role, HIF-1α has been reported to directly upregulate Noxa expression through the hypoxia response element on its promoter (Kim *et al.*, 2004).

Although not directly addressed in this study, the contribution of reactive oxygen species as mediators of this $p38^{MAPK}$/HIF-1 cascade is very likely. As indicated in our previous work (Van Laethem *et al.*, 2006), reactive oxygen species are required to trigger the apoptosis signaling kinase 1 (Ask-1)/$p38^{MAPK}$ cascade in UVB-treated NHKs and HIF-1 is generally thought to be a redox-sensitive transcription factor (Pouyssegur and Mechta-Grigoriou, 2006). However, how $p38^{MAPK}$ regulates HIF-1 is still unclear, although several possibilities can be proposed. For example, $p38^{MAPK}$ could directly phosphorylate HIF-1α to stabilize and/or transactivate the transcription factor (Kwon *et al.*, 2005; Rezvani *et al.*, 2007). Alternatively, $p38^{MAPK}$ could regulate HIF-1α at post-transcriptional and translational levels, by stabilizing its mRNA, or through the activation of Siah2, an E3 ligase that targets PHD3, a negative regulator of HIF-1α, for proteasomal degradation (Khurana *et al.*, 2006).

Together with $p38^{MAPK}$ also JNK is stimulated after UVB (Assefa *et al.*, 2005) and could contribute to the pro-apoptotic HIF-1/Noxa cascade. However, in previous studies, we found that $p38^{MAPK}\alpha^{-/-}$ mouse embryonal fibroblasts are protected from UVB-induced apoptosis even if JNK activation is fully preserved. Moreover, the killing effect of Ask-1 overexpression, an upstream MAPKKK of both $p38^{MAPK}$ and JNK, is almost completely reversed by $p38^{MAPK}$ inhibition alone in UVB-irradiated NHKs (Van Laethem *et al.*, 2006).

Irrespective of the exact molecular mechanism involved, this study underscores that Noxa is a crucial executor of the pro-apoptotic signal initiated by the $p38^{MAPK}$ cascade in UVB-treated NHKs. It has been recently proposed that Noxa exerts its pro-apoptotic function by degrading and neutralizing Mcl-1 (Ploner *et al.*, 2008), which is a major survival protein required for normal keratinocyte differentiation (Sitailo *et al.*, 2009). This study confirms this assumption, because Noxa knockdown (or $p38^{MAPK}$ inhibition) results in the maintenance of Mcl-1 levels after UVB, an effect that is functionally associated with a significant increase in cytoprotection. Although the underlying mechanisms of Noxa-promoted degradation of Mcl-1 are not completely understood, blocking Noxa induction in UVB-treated NHKs inhibits Bax activation and intrinsic apoptosis. Whether Mcl-1 degradation itself or rather the subsequent Bim (Bcl-2-interacting mediator of cell death) release is responsible for Bax activation (Han *et al.*, 2007) is still under investigation.

All together this study shows that NHKs exposed to killing doses of UVB, through a signaling pathway governed by $p38^{MAPK}$, engage mitochondrial apoptosis through the induction of Noxa. This pathway requires HIF-1, whereas p53 is largely dispensable. This is an important notion because p53 is frequently mutated in sun-exposed skin cells and prompts future studies on the role of the $p38^{MAPK}$/Noxa pathway in photocarcinogenesis.

MATERIALS AND METHODS

Materials

Anti-Mcl-1 and anti-PUMA antibodies were from Cell Signaling (Beverly, MA). For the detection of Noxa, we purchased a monoclonal antibody from Imgenex (San Diego, CA) for immunocytofluorescence and used antibody clone 114C307 from Abcam (Cambridge, UK) for western blot analysis. Anti-BNIP3 was from Abcam. siRNA SMARTpool for $p38^{MAPK}$, p53, Noxa, HIF-1α, or the siCONTROL nontargeting Scrambled Pool was from Dharmacon (Thermo Fisher Scientific, Waltham, MA). Actinomycin D and cycloheximide were from Sigma (Saint Louis, MO). The source of other materials used in this study has been described in previous work (Van Laethem *et al.*, 2006).

Cells, culture conditions, and treatments

Human primary keratinocytes were derived from foreskin of young donors (less than 1 year of age) as previously described (Gilchrest, 1983). Keratinocyte culturing conditions, transduction of the SCC cell line A253 and UVB irradiation, have been described in previous studies (Courtois *et al.*, 1997; Van Laethem *et al.*, 2006).

Western blot analysis, cellular fractionation, and immunoprecipitation

After UVB irradiation, we prepared cell lysates as reported in previous studies (Van Laethem *et al.*, 2006). For HIF-1α detection, we added PhosStop phosphatase cocktail (Roche Applied Science, Basel, Switzerland) to the lysis buffer. Cellular fractionation to recover cytosolic and particulate fractions was performed as already described (Van Laethem *et al.*, 2006). Protein concentration was determined using BCA assay (Perbio, Thermo Fisher Scientific, Waltham, MA). Samples with loading buffer were prepared and processed on the Criterion system (Bio-Rad Laboratories, Hercules, CA) on a 4–12% Bis-Tris gel and Protran 2 μm-pored nitrocellulose paper (PerkinElmer, Wellesley, MA). The activated conformation of Bax was immunoprecipitated from protein lysates (4 μg of antibody per sample), after initial preclearing with protein A-sepharose beads and further processed on the Criterion system (Bio-Rad Laboratories) as described above.

RNA extraction, cDNA synthesis, and real-time PCR

Total RNA was extracted using the PureLink RNA Mini kit from Invitrogen (Carlsbad, CA) and subsequently reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada). Quantitative real-time PCR assays were performed as described by Flamant *et al.* (2009). The sequences of the primers (Eurogentec, Liège, Belgium) are available on request.

Cell death assays

For apoptosis detection, we used both Cell Death Detection *ELISA*^{plus} (Roche Applied Science) performed according to manufacturer's protocol and FACS Calibur (BD Biosciences, San Diego, CA) analysis of SubG1 fraction (Van Laethem *et al.*, 2006).

HIF-1 DNA-binding assay

TransAM ELISA kit (Active Motif, Carlsbad, CA) for detecting DNA-binding activity of HIF-1 was performed according to manufacturer's recommendations.

Immunocytofluorescence

Cells were prepared for analysis as described before (Van laethem *et al.*, 2006). Images were acquired using an Olympus (Melville, NY) FluoView FV1000 confocal microscope.

siRNA transfection

Cells were transfected by adding 400 µl Keratinocyte-SFM without growth factors with 6 µl Dharmafect 1 (Dharmacon, Thermo Fisher Scientific) and a final concentration of 80 nM siRNA (Dharmacon, Thermo Fisher Scientific) to 10 cm dishes with 2 ml growth factor-containing Keratinocyte-SFM. Experiments were performed 72 h after transfection when maximal knockdown occurred.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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